Changes in Essential Oil Composition and Leaf traits of Basil (*Ocimum basilicum* L.) Affected by Bio-stimulators / fertilizers Application

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Abstract

Background: Basil (*Ocimum basilicum* L.), a member of the Lamiaceae family, has traditionally been used as a medicinal plant in the treatment of headaches, coughs, diarrhea, constipation, warts, worms, and kidney malfunctions.

Objective: To investigate the foliar application effects of bio-stimulators and bio-fertilizers on morphological and phytochemical traits of basil (*Ocimum basilicum* L.).

Methods: Bio-stimulators in three commercial formulations of aminolforte, kadostim and fosnutren (with concentration of 1.5 L.ha^{-1}) through foliar application, and bio-fertilizers in three commercial formulations of nitroxin, super-nitro plus and barvar II (with concentration of 0.5 L.ha^{-1}) through seed inoculation were considered as two studied factors.

Results: The results showed that the interaction effect of bio-stimulators and bio - fertilizers was significant ($p<0.01$) on all of studied parameters except of chlorophyll content (SPAD value). The highest leaf fresh weight (25.47 g/plant) and leaf dry weight (6.48 g/plant) were obtained under fosnutren and nitroxin treatment, also maximum leaf number (206.33) was recorded in aminolforte and nitroxin treatment. The highest leaf area (1302.2 mm^2/plant) was observed in kadostim and nitroxin treatment. Also results showed that the highest content of essential oil (0.43%) was obtained in aminolforte and nitroxin, methyl chavicol (37.13%) in fosnutren and super-nitro plus, geranial (29.05%) and caryophylene (6.66%) in kadostim and nitroxin, and carvacrol (31.60%) in fosnutren and nitroxin treated plants.

Conclusion: In general, the best treatment to improve growth and phytochemical traits of *Ocimum basilicum* were kadostim×nitroxin and fosnutren×nitroxin.

Keywords: ***Ocimum basilicum* L., Bio-fertilizers, Bio - stimulators, Essential Oil, Leaf traits**

Introduction

Basil (*Ocimum basilicum* L.), a member of the Lamiaceae family, is used both as a culinary and ornamental herb [1-3]. The genus *Ocimum* contains between 50 and 150 species of herbs and shrubs found in the tropical regions of Asia, Africa, and Central and South America [4, 5]. Traditionally, basil has been used as a medicinal plant in the treatment of headaches, coughs, diarrhea, constipation, warts, worms, and kidney malfunctions [6]. Externally, basil can be used as an ointment for insect bites, and its oil is applied directly on the skin to treat acne [7]. Natural components from basil have long been used to flavor foods and dental and oral products [6, 8]. Iranian basils are used to treat fevers, throat congestions, and stomachache [9, 10]. These activities are chiefly attributed to a variety of phenolic compounds and composition of essential oil. The main compounds responsible for typical basil aroma are chavicol methyl ether (estragol), linalool, eugenol, 1, 8-cineole and methyl cinnamate [31]. On the basis of more than 200 components of essential oils isolated from *O. basilicum* L. Lawrence (1988) classified four major chemotypes of basil: (1) methyl chavicol-rich, (2) linalool-rich, (3) methyleugenol-rich, (4) methyl cinnamate-rich, and also numerous subtypes.

Extensive attempts are being conducted to find suitable solutions for improving the soil quality, agricultural products and polluters' elimination. Decrease of these environmental threats in line with increase of crops yield needs application of new agricultural techniques. One of these techniques is application of bio-stimulators that is purification of proteins/ amino acid compounds from natural sources and alteration to specific oligopeptides [11, 12]. Bio-stimulators as biological substances stimulate metabolism and metabolic processes to increase plants yield. These compounds like commercial formulations of aminolforte, kadostim and fosnutren have the basis of amino acid and they improve quantitative and qualitative growth [13]. The positive effect of bio-stimulators on production, quality and growth of vegetables, *Camellia* species and forage crops is previously reported [14].

Bio-fertilizers are fertilizing compounds that are composed of one or more species of useful soil-living organisms and are presented on preservative substances. Bio-fertilizers are introduced as microbial inoculation stock as a compound with effective microbial strains and with high yield of supplying one or more nutritional elements. Bio-fertilizers are micro-organisms that are able to change nutritional elements from useless form to effective and useful compounds and this change is conducted in a biological process. Production expenses of bio-fertilizers are low and it does not cause pollution in the environment [15]. Yousef et al. (2004) reported that biological fertilizers composed of micro-organisms and replacement of them with artificial growth regulators improve growth characteristics and essential oil compounds of *Salvia officinalis*. Also, application of *Pseudomonas fluorescens* increased yield of *Catharanthus roseus* [16]. However, the aim of this study was to investigate the effects of bio-stimulators and bio-fertilizers on morphological and phytochemical traits of basil (*Ocimum bacilicum* L.).

Materials and Methods

This experiment was carried out in 2011-2012 at Iranian Academic Centre for

Education, Culture & Research (ACECR), Institute of Medicinal Plants (56° 35´ N and 50° 58´ E; 1500 m elevation). The soil was loam-silt with 0.071% N, 48.9 $mg.kg^{-1}$ Phosphorous, 33.6 $mg.kg^{-1}$ Potassium, EC 2.71 $dS.m^{-1}$, and pH 8.3.

In this study, a factorial experiment was conducted on the basis of randomized complete blocks design with 16 treatment and 3 replications. Seed inoculation of commercial formulations of bio-fertilizers including nitroxin, super-nitro plus and barvar II (500 ml in 5 Kg seed) and control treatment, along with foliar application of bio-stimulators in commercial formulations of aminolforte, kadostim and fosnutren (at the concentration of 1.5 $L.ha^{-1}$) and control treatment (without application of bio-stimulators) were applied in this experiment.

The commercial formulations of bio-stimulators were supplied by Inagrosa Industries Agro Biologicals, Madrid, Spain. The details of the formulations are given in Table 1. All of the treatments were sprayed in four growth stages including: three, four and five weeks after planting and in flowering stage.

Super-nitro plus is composed of different species of N stabilizing bacteria, controllers of soil-living pathogens and growth stimulating bacteria (PGPR) like *Basillus subtilis*, *Azospirillum* spp., and *Pseudomonas* spp. Nitroxin is composed of *Pseudomonas* genus. Concentration of nitrogen stabilizing and growth stimulators bacteria in super-nitro plus and nitroxin is 10^8 living cells (CFU). Barvar II is from phosphate solvent bacteria and different genera of *Pseudomonas*/*Bacillus*. Number of living cells (CFU) was minimum 10^7 living cells from each of bacteria genera in per ml of bio-fertilizer, which are composed of different genera of phosphorous solvent bacteria. These bacteria have the ability of production of mineral and organic acids and phosphatise enzyme secretion and in this way it will change the sources of mineral and organic phosphorous in soil to useful form in plant.

Twenty seeds were sown at each pot and five plants were remained in each pot after thinning. Other operations were done regularly during the growing season. Studied parameters

Table 1- Formulation of bio-stimulators used in the experimental treatments

Biostimulators*	Formulation of compounds **
Aminolforte	Free amino acids 3750 $mg.L^{-1}$, organic components 2% and total N 1.1% (urea N 0.8% and organic N 0.3%)
Kadostim	Free amino acids 3750 $mg.L^{-1}$, organic components 2% and total N 4.2% (amonia N 0.8%, nitric N 3.1% and organic N 0.3%) and potassium (K_2O) 6%
Humiforte	Free amino acids 3750 $mg.L^{-1}$, organic components 2% and total N 6% (amonia N 1.4%, urea N 3.7%, nitric N 0.5% and organic N 0.3%), potassium (K_2O) 5% and phosphorous (P_2O_5) 3%
Fosnutren	Free amino acids 3750 $mg.L^{-1}$, organic components 2% and total N 3.8% (amonia N 2.1%, nitric N 1.4% and organic N 0.3%) And phosphorous (P_2O_5) 6%

* Biostimulators supplied by Inagrosa Industries Agro Biologicals are compatible to the climate of Iran.

** Quantity and kind of free amino acids applied in the formulation of bio-stimulators in this experiment based on the percent of total amino acids are as follows: Glysin 11.2%, Valine 5.1%, Proline 8.3%, Alanin 13.2%, Aspartic acid 4.4%, Arginine 8.3%, Glutamic acid 0.9%, Lysine 5.1%, Lucine 16.4%, Isolucine 4.4%, Phenylalanin 5.1%, Methionine 4.2%, Serin 3.9%,Treonine 0.3%, Histidine 0.3%, Tyrosine 1.5%, Glutamine 0.9%, Systein 0.3%, Aspargine 0.4%, Tryptophan 0.4%.

were leaf fresh and dry weight (g), leaf number, chlorophyll content (SPAD Value), leaf area, essential oil content (% EO) and composition. In order to measuring dry matter, the plants material was placed in the electric oven of 75°C until the constant weight was gained. For measurement of leaf chlorophyll content (SPAD value), five leaves of each plant were selected and mean of leaf chlorophyll content (SPAD value) was measured by device of SPAD (Minolta, 50 II, JAPAN).

Essential oils of the aerial parts were extracted by hydrodistillation method for 3 h using clevenger-type apparatus [17]. The oils were dried over anhydrous sodium sulphate and kept at −4 °C until it was analyzed.

GC analysis was carried out on a Younglin Instrument Acme 6000 M gas chromatograph equipped with Flame Ionization Detector (FID) and a HP-5 capillary column (30 m×0.25 mm; 0.25 μm film thicknesses). The oven temperature was held at 50ºC for 5 minutes, and then programmed at 3ºC min^{-1} to 240ºC and after that programmed at 15ºC min^{-1} to 300ºC (held for 3 minutes). Other operating conditions were: carrier gas, He with a flow rate of 0.8 mL min-1; injector and detector temperatures was 290ºC, and split ratio, 1:10. GC/MS analysis was performed on a GC mentioned above coupled with a Agilent Technologies 5973 Mass system. The other operating conditions were the same conditions as described above, mass spectra were taken at 70 eV. Mass range was from m/z 35 – 375 amu. Quantitative data were obtained from the electronic integration of the FID peak areas. The components of the essential oils were identified by comparison of their mass spectra and retention indices with those published in the literature [18, 19] and presented in the MS computer library. Each analysis was performed in triplicate.

Analysis of variance of the results was done using the SPSS software (ver. 17), and means in the results were compared by "Duncan's multiple range test" at $p<0.01$.

Results

The results indicated that interaction effect of bio-stimulators and bio-fertilizers had significant effect on leaf fresh weight, leaf dry weight, leaf number, leaf area, essential oil, methyl chavicol, geranial, carvacrol and caryophylene ($p\leq 0.01$).

Main and interaction effects of bio-stimulators and bio-fertilizers were non-significant on chlorophyll content (Table 2).

Concerning mean comparisons, the maximum leaf fresh weight (25.47g/plant) was obtained in fosnutren and nitroxin treated plants. However, the lowest leaf fresh weight (5.59 g/plant) was observed in plants treated with fosnutrn and barvar II (bio-phosphor). The results indicated that the most leaf dry weight (6.48 g/plant) was obtained in fosnutren and nitroxin treatments. The least leaf dry weight (0.81 g/plant) was observed by application of fosnutren and barvar II. The results showed that the maximum leaf number (98.33) was obtained in aminolforte and nitroxin treated plants. While, the minimum leaf number (49.66) was observed under control and barvar II treatments. The highest leaf area (902.3 mm^2/plant) was obtained in kadostim and nitroxin treated plants (Table 3).

Considering interaction effect of bio-stimulators and bio-fertilizers, the highest amount of essential oil (0.43%) was obtained in aminolforte and nitroxin treatment. The highest content of methyl chavicol (37.13%)

Table 2 - Analysis of variance for measured traits in sweet basil (*Ocimum basilicum* L.)

S.O.V	DF	Mean Squares									
		Leaf fresh weight	Leaf dry weight	Leaf number	Leaf area	Chlorophyll content	Essential oils	Methyl chavicol	Geranial	Carvacrol	Cryophylene
Block	2	4.866	0.524	451.937	39894.119	12.608	0.020	211.75	189.20	54.57	7.391
Bio-stimulator	3	77.759**	4.292**	6193.833**	26664.2**	37.482^{ns}	0.005**	12.815*	8.941^{ns}	9.592^{ns}	1.911**
Bio-fertilizer	3	42.731**	2.323**	1745.833**	46749.5**	43.363^{ns}	0.000002^{ns}	40.02**	59.274**	24.125*	3.465**
S×F	9	98.512**	12.425**	7068.704**	606515**	20.898^{ns}	0.033**	270.74**	21.21**	54.748**	3.110**
Error	30	7.052	0.324	216.893	5439.015	15.011	0.001	3.970	6.098	7.999	0.369
Cv		17.57	19.91	14.87	13.87	10.37	12.44	10.17	11.56	10.77	14.99

*, ** and ^{ns} shows significant at 5%, 1%, and nonsignificant, respectively.

Table 3 - Mean comparisons* for interaction effects of bio-stimulators and bio-fertilizers on measured parameters of basil (*Ocimum bacilicum* L.)

Treatments		Leaf fresh weight (g/plant)	Leaf dry weight (g/plant)	Leaf number per plant	Leaf area (mm^2/plant)	Chlorophyll content (SPAD value)	Essential oils (%)	Methyl chavicol (%)	Geranial (%)	Carvacrol (%)	Caryophylene (%)
Control	Control	15.51^{cd}	3.17^{c}	78^{d}	650^{c}	31.53	0.23^{c}	23.50^{cd}	16.51^{f}	19.61^{c}	2.70^{ef}
	Nitroxin	10.60^{ef}	1.41^{ef}	78.66^{d}	307.58^{fgh}	37.10	0.23^{c}	18.80^{ef}	20.85^{bcdef}	24.10^{cde}	4.23^{bcd}
	Super-nitro plus	13^{def}	1.79^{ef}	98.66^{cd}	426.52^{ef}	35.53	0.23^{c}	16.26^{fg}	23.71^{bcd}	31.22^{a}	3.41^{cdef}
	Barvar II (Biophosphor)	10.07^{fg}	1.87^{def}	49.66^{f}	424.70^{ef}	35.43	0.23^{c}	14.03^{g}	20.28^{bcdef}	24.79^{bcde}	4.33^{bc}
Aminol forte	Control	10.06^{fg}	3.20^{c}	48.66^{ef}	184.85^{gh}	35.66	0.23^{c}	27.73^{b}	22.61^{bcde}	27.63^{abc}	4.96^{b}
	Nitroxin	19.25^{bc}	2.33^{cde}	98.33^{a}	494.70^{de}	43.86	0.43^{a}	16.20^{fg}	23.90^{bc}	30.00^{ab}	4.23^{bcd}
	Super-nitro plus	15.74^{cd}	2^{de}	72^{de}	459.09	38.63	0.23^{c}	21.27^{de}	21.69^{bcde}	24.16^{cde}	3.35^{cdef}
	Barvar II (Biophosphor)	17.73^{cd}	5.05^{b}	81.66^{d}	845.2^{a}	38.13	0.23^{c}	14.24^{g}	18.07^{ef}	25.30^{bcd}	3.83^{bcde}
Kadostim	Control	18.43^{c}	1.45^{ef}	85^{cd}	309.39^{fgh}	38.30	0.35^{b}	14.37^{g}	19.01^{def}	30.10^{ab}	3.80^{bcde}
	Nitroxin	15.03^{cde}	3.11^{c}	86.66^{cd}	902.3^{a}	41.23	0.16^{d}	28.73^{b}	29.05^{a}	26.88^{abcd}	6.66^{a}
	Super-nitro plus	16.61^{cd}	6.28^{a}	90.67^{b}	171.61^{h}	38.93	0.36^{b}	13.91^{g}	20.12^{bcdef}	27.12^{abcd}	4.83^{b}
	Barvar II (Biophosphor)	23.16^{ab}	2.90^{cd}	96.33^{a}	286.06^{gh}	31.53	0.16^{d}	25.43^{bc}	21.43^{bcde}	22.81^{cde}	3.07^{def}
Fosnutren	Control	8.97^{fg}	1.47^{ef}	89^{bc}	583.33^{d}	38.26	0.20^{cd}	6.09^{h}	21.00^{bcdef}	21.77^{de}	2.66^{f}
	Nitroxin	25.47^{a}	6.48^{a}	75^{d}	315.91^{fg}	37.66	0.20^{cd}	14.28^{g}	24.40^{b}	31.60^{a}	3.99^{bcd}
	Super-nitro plus	16.52^{cd}	2.06^{de}	87^{cd}	749.24^{b}	37.20	0.20^{cd}	37.13^{a}	19.23^{cdef}	23.11^{cde}	4.80^{b}
	Barvar II (Biophosphor)	5.59^{g}	0.81^{f}	83^{d}	270.91^{gh}	38.70	0.40^{a}	21.30^{de}	19.83^{bcdef}	30.10^{ab}	3.95^{bcd}

*Values in a column bearing different superscript are significantly different at 0.01 levels

was observed by fosnutren and super-nitro plus. According to mean comparisons, the most amounts of geranial (29.05%) and caryophylene (6.66%) was obtained in kadostim and nitroxin treated plants. The highest amount of carvacrol (31.60%) was obtained under fosnutren and nitroxin treatments (Table 3).

Discussion

The results indicated that the bio-stimulators and bio-fertilizers and their interaction had significant effect ($p<0.01$) on fresh and dry weight of leaves. These results are according to Abo-Dahab and Abd El-Aziz (2006) research on *Philodendron erubescens*. The data recorded in the two seasons showed that the amino acids diphenylamine and tryptophan significantly increased the fresh and dry weights of the different parts of the plant (leaves, stem, roots and the whole plants), compared to those of the control plants. The increase in the fresh and dry weights as a result of the tryptophan treatments may be due to its conversion into IAA [21]. Bacteria of *Pseudomonas* and *Basillus* change insoluble phosphorous to soluble form and in other words they are phosphate dissolving bacteria [22]. Spices of *Pseudomonas fluorescens* with different mechanisms like synthesis of anti-biotic, growth regulators and enzymes regulating ethylene synthesis in plant improve the plant growth [23].

Bio-stimulators and bio-fertilizers and their interaction had significant effect ($p<0.01$) on leaves number. Previously, Sanchez et al. (2005) reported that application of biological fertilizers could be increased yield of *Matricaria recutita* L. Our results are not in according to the results of Abdel-Mawgoud et al. (2011) study. They reported that the lowest concentration of amino-green causes an increase in number of leaves. Of course, the our results are in line with that of Ayman et al. (2009) experiment on *Vicia faba* L. concerning interaction effect of humic acid and amino acid in isolation and in presence of chelated micro nutrients and the results by Shekari et al. (2012) on *Plantago psyllium* L. Increase in yield and growth parameters is proved to be feasible using amino acids. Therefore, supply of nutritious sources to form protein tissue is essential [28].

Although, the main and interaction effects of bio-stimulators and bio-fertilizers on leaves chlorophyll content (SPAD value) wasn't statistically significant, the bio-stimulators and bio-fertilizers and their interaction had significant effect ($p<0.01$) on the leaf area. These results are in line with Nahed et al (2010) study on use of amino acids tirozin, thiamin and tryptophan on *Thuja orientalis* L. They concluded that all growth parameters improved with increase in concentration of amino acids. Positive effect of amino acids on yield might be due to stimulating effect of amino acids on plant cells growth. However, amino acids were introduced by Goss (1973) as a source of energy during lack of carbohydrates.

Although, the main effect of bio-stimulators on geranial and carvacrol wasn't significant, the main effect of bio-fertilizers on geranial and carvacrol were significant. Of course, the interaction effect of bio-stimulators and bio-fertilizers was significant on chavicol, Geranial and Carvacrol. These results are in line with results of Franz (1983) study on *Matricaria recutita* L. plants with application of Nitrogen fertilizer. The results showed that

nitrogen fertilizer increases essential oil content and the nutrition affects on synthesis of essential oil indirectly. It resulted that essence content increased with increase in nitrogen or phosphorous fertilizer and it decreased with application of potassium fertilizer.

Fatma et al., (2006) in a greenhouse experiment on *Origanum vulgare* L. showed that biological fertilizers like *Azospirillum* and *Azetobacter* and phosphate solvent bacteria had considerable effects on growth parameters and amount of essential oil.

Conclusion

In this experiment, commercial formulation of bio-stimulators and bio-fertilizers had significantly positive effect on growth and phytochemical parameters of basil (*Ocimum bacilicum* L.). Due to existence of amino acids, the bio-stimulators could be promote growth and secondary metabolite production. Bio-fertilizers as compounds with effective microbial strains and high yield of supplying one or more nutritional elements improved the growth parameters. The interaction effects of bio-stimulators and bio-fertilizers improved growth and phytochemical traits. These results could be due to ability of these bio-compounds in supply essential nutrients like nitrogen, phosphorous and potassium and subsequently their direct effects on morphological and phytochemical traits of the plant.

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